

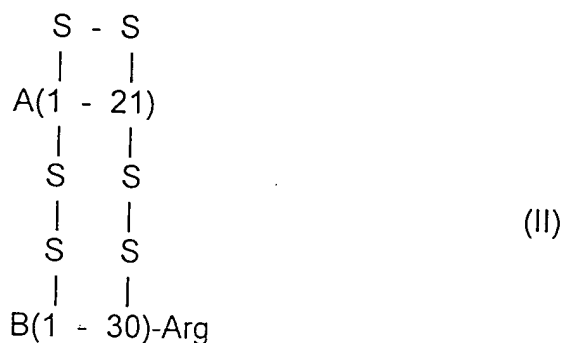
APPENDIX A
PENDING CLAIMS

U.S. Patent Application No. 08/402,394

Filed: March 10, 1995

Inventors: Michael DORSCHUG et al.

21. (Thrice Amended) A method for the preparation of a mono-Arg-insulin compound of formula II



in which A(1-21) and B(1-30) denote the A and B chains of human insulin and the -S-S- bridges are positioned as in insulin, which comprises:

(a) expressing in a bacterium a DNA molecule encoding a fusion protein which comprises a mini-proinsulin compound of the formula:



- (b) liberating said mini-proinsulin compound from said fusion protein;
- (c) folding and forming disulfide bridges in said mini-proinsulin compound;
- (d) incubating said mini-proinsulin compound with trypsin; and
- (e) precipitating the mono-Arg-insulin.

22. (Twice Amended) A method for the preparation of insulin which comprises:

(a) expressing in a bacterium a DNA molecule encoding a fusion protein

which comprises a mini-proinsulin compound of the formula:



in which B(1-30) and A(1-21) denote the B and A chains of insulin;

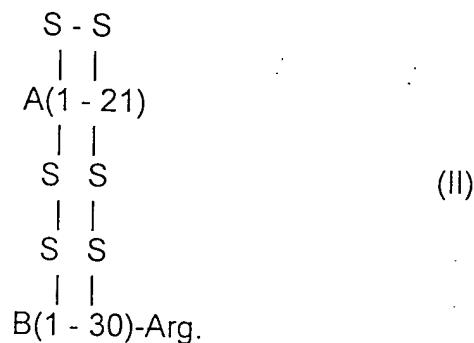
(b) liberating said mini-proinsulin compound from said fusion protein;

(c) folding and forming disulfide bridges in said mini-proinsulin compound;

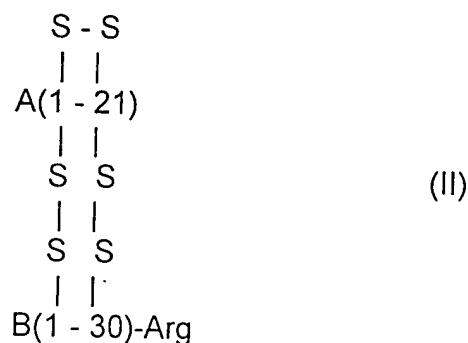
(d) simultaneously incubating said mini-proinsulin compound with trypsin and carboxypeptidase B; and

(e) precipitating the insulin.

23. A method as claimed in claim 22, wherein step (d) is carried out in one vessel without having to isolate as an intermediate mono-Arg-insulin of formula II



25. (Amended) A method for the preparation of a mono-Arg-insulin compound of formula II

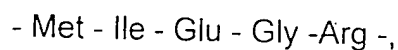


in which A(1-21) and B(1-30) denote the A and B chains of human insulin and the -S-S- bridges are positioned as in insulin, which comprises:

(a) expressing in a bacterium a DNA molecule encoding a fusion protein which comprises



bonded via a bridging member,



to a peptide which stabilizes the fusion protein;

(b) liberating a mini-proinsulin compound from said fusion protein by cleaving the expressed fusion protein resulting from step (a) with cyanogen bromide;

(c) folding and forming disulfide bridges in said mini-proinsulin compound;

(d) incubating said mini-proinsulin compound with trypsin; and

(e) precipitating the mono-Arg-insulin.

26. (Amended) A method for the preparation of insulin which comprises:

(a) expressing in a bacterium a DNA molecule encoding a fusion protein which comprises

B(1-30)-Arg-A(1-21)

bonded via a bridging member,

- - Met - Ile - Glu - Gly -Arg - ,

to a peptide which stabilizes the fusion protein;

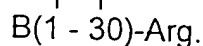
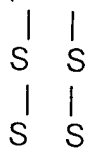
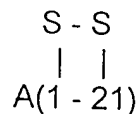
(b) liberating a mini-proinsulin compound from said fusion protein by cleaving the expressed fusion protein resulting from step (a) with cyanogen bromide;

(c) folding and forming disulfide bridges in said mini-proinsulin compound;

(d) simultaneously incubating said mini-proinsulin compound with trypsin and carboxypeptidase B; and

(e) precipitating the insulin.

27. A method as claimed in claim 26, wherein step (d) is carried out in one vessel without having to isolate as an intermediate mono-Arg-insulin of the formula II



(II)

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31. (Amended) A method for the preparation of insulin, without formation of substantial amounts of insulin Des-B30, comprising:

(a) expressing in a bacterium a DNA molecule encoding a fusion protein which comprises

B(1-30) - Arg - A(1-21)

bonded via a bridging member,

-Met-Ile-Glu-Gly-Arg-,

to a peptide which stabilizes the fusion protein;

(b) liberating a mini-proinsulin compound from said fusion protein by cleaving the expressed fusion resulting from step (a) with cyanogen bromide to produce mini-proinsulin;

(c) incubating the product formed in step (b) with sodium tetrathionate to form hexa-5-sulfonate;

(d) simultaneously incubating the S-sulfonate mini-proinsulin formed in step (c) with trypsin and carboxypeptidase; and

(e) precipitating the insulin.

33. A compound of the formula I

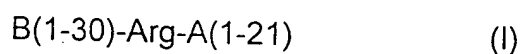
B(1-30)-Arg-A(1-21) (I)

wherein A(1-21) and B(1-30) denote the A and B chains of human insulin.

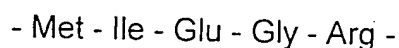
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34. A nucleic acid sequence encoding the compound of formula I as claimed in claim 33.
35. A vector comprising the nucleic acid sequence of claim 34.
36. A host cell containing the nucleic acid sequence of claim 34.
37. A fusion protein comprising a compound of the formula I

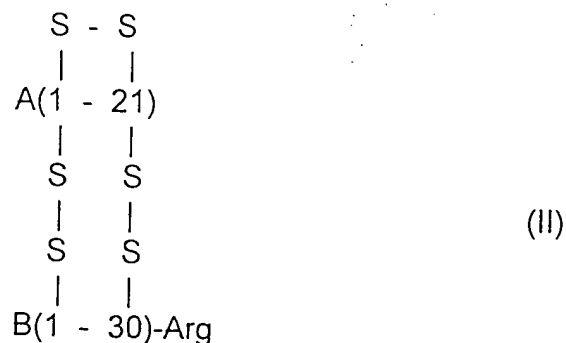


wherein A(1-21) and B(1-30) denote the A and B chains of human insulin, and wherein the compound is bonded via a bridging member



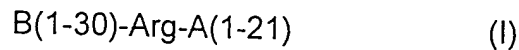
to a peptide which stabilizes the fusion protein.

38. A process for the preparation a compound as claimed in claim 33, which comprises:
- a) expressing a DNA sequence encoding the compound of the formula I in a bacterium; and
- b) when the DNA sequence encodes a fusion protein, liberating the compound of formula I from the fusion protein.
39. A method for the preparation of a compound of the formula II



wherein A(1-21) and B(1-30) denote the A and B chains of human insulin and the -S-S-bridges are positioned as in insulin, comprising:

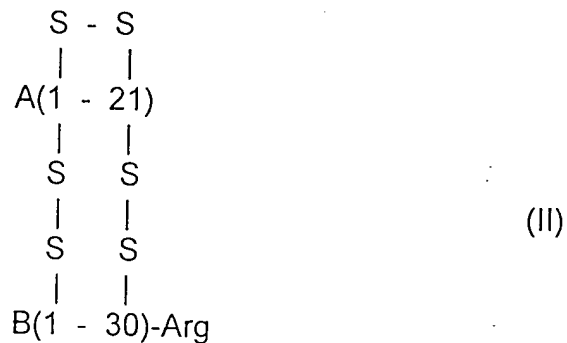
- a) expressing a DNA sequence encoding the compound of formula I



in a bacterium; and

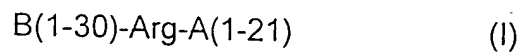
- b) cleaving the expressed compound of step (a) with trypsin.

40. A method for the preparation of a compound of the formula II



wherein A(1-21) and B(1-30) denote the A and B chains of human insulin and the -S-S-bridges are positioned as in insulin, comprising

- a) expressing a DNA sequence encoding the compound of formula I



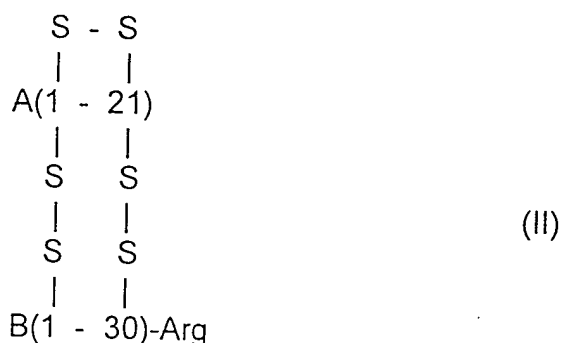
in a bacterium;

- b) cleaving the expressed compound of step (a) with trypsin resulting in the compound of the formula II; and

(c) cleaving the resulting compound of step (b) with carboxypeptidase B.

41. The method of claim 40, wherein steps (b) and (c) are carried out in one vessel without having to isolate the intermediate compound of the formula II.

42. A method for the preparation of a mono-Arg-insulin compound of the formula II



in which A(1-21) and B(1-30) denote the A and B chains of human insulin and the -S-S- bridges are positioned as in insulin, which comprises:

(a) expressing a DNA sequence encoding a mini-proinsulin compound of the formula:



in a yeast; and

(b) cleaving said mini-proinsulin compound with trypsin.

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